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α_{2u}-Globulin Nephropathy: Review of the Cellular and Molecular Mechanisms Involved and Their Implications for Human Risk Assessment

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This paper reviews what is known about the induction of α_{2u}-globulin nephropathy and carcinogenesis. This unique male-rat-specific disease is associated with exposure to an ever-increasing number of chemicals. The processes leading to nephropathy and renal cancer are among the best-understood mechanisms for nongenotoxic chemicals and strongly support that it is a male-rat-specific process that is not relevant for human risk assessment. Nevertheless, the data available for individual chemicals vary greatly. This necessitates a case-by-case analysis of the available data when determining the relevance for humans of this chemically induced renal disease in male rats.

Introduction

After the discovery that unleaded gasoline (UG) caused kidney tumors in male rats but not in female rats or either sex of mice, a series of studies was undertaken to understand this sex- and species-specific disease. Investigations by Halder et al. demonstrated the formation of protein droplet nephropathy by a series of branched aliphatic hydrocarbons (*1,2*). One of the most potent hydrocarbons causing this disease was 2,2,4-trimethylpentane (TMP), an important component of gasoline. The suggestion that hydrocarbon nephropathy might be related to the male-rat-specific protein, α_{2u}-globulin (α_{2u}) was first made by Alden, who identified α_{2u} as the accumulating protein in male rats exposed to decalin and hypothesized that a similar phenomenon might occur with other hydrocarbons (*3,4*). Since these early studies, many additional chemicals have been shown to cause α_{2u} nephropathy in male rats. None of these have caused a similar nephropathy in female rats or either sex of any other species (*5–7*). Thus, α_{2u} nephropathy appears to represent a sex- and species-specific disease.

α_{2u} Nephropathy is characterized by the accumulation of protein droplets in the P2 segment of the proximal tubule, subsequent single cell necrosis, the formation of granular casts at the junction of the proximal tubule and the thin loop of Henle, and the presence of regenerative tubules. With chronic exposure, there is a progression of these lesions, the formation of linear mineralization in the renal medulla, and an exacerbation of chronic progressive nephropathy, the spontaneous nephropathy of aging rats. Chronic exposure of male rats to UG and numerous other nongenotoxic chemicals also led to the formation of exposure-related increases in renal tumors. The incidence of tubular neoplasms of the kidney induced by agents causing α_{2u} nephropathy ranged from 0 to 26%. This is markedly different from the induction of renal tumors by known genotoxic agents, which frequently reaches 100% (*8*). The chemicals known to cause this α_{2u} nephropathy are listed in Table 1.

The protein α_{2u} is a well-characterized, low molecular weight protein of 18,700 D that is synthesized in the liver of male rats under androgenic control, secreted into the plasma, and freely filtered by the glomerulus (*9,10*). Approximately half of the α_{2u} present in the glomerular filtrate is resorbed by the P2 segment of the proximal tubule of the nephron, and the other half is excreted in the urine (*11,12*). Tubular resorption of α_{2u} occurs via endocytosis. The endosomes then fuse with primary lysosomes, where the protein undergoes hydrolytic digestion.

Whereas large amounts of protein are found in the urine of male rats, α_{2u} is found in the urine of female and sexually immature rats at concentrations that are less than 1/100 that found in young adult males (*13,14*). Hepatic synthesis accounts for most of the α_{2u} in male rats, with synthesis beginning at the onset of sexual maturity and increasing to 20 weeks of age, after which it plateaus and then begins to decline with increasing age (*15*). Female rats do not

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Table 1. Data sets for chemicals causing α_{2u}-globulin nephropathy (7).

Substance/chemical	Protein droplets	Increased α_{2u}	α_{2u} Binding	Cell proliferation	Initiation/ promotion
d-Limonene	+	+	+	+	+
Unleaded gasoline	+	+	+	+	+
2,2,4-Trimethylpentane	+	+	+	+	+
1,4-Dichlorobenzene	+	+	+	+	NR
Isophorone	+	+	+	+	NR
3,5,5-Trimethyl hexanoic acid derivatives	+	+	+	NR	NR
Decalin	+	+	NR	+[a]	NR
Tetrachloroethylene	+	+	NR	+	NR
Pentachloroethane	+	+	NR	+	NR
C_{10}–C_{12} isoparaffinic solvent (saturated aliphatic hydrocarbons)	+	+	NR	NR	NR
Lindane	+	+	NR	NR	NR
BW540C	+	+	NR	NR	NR
BW58C	+	+	NR	NR	NR
Levamisole	+	+	NR	NR	NR
Gabapentin	+	+	NR	NR	NR
Tridecyl acetate	+	+	NR	NR	NR
Isopropylcyclohexane	+	+	NR	NR	NR
JP-5 jet fuel (mixed distillate hydrocarbons)	+	NR	NR	NR	NR
JP-4 jet fuel (mixed distillate hydrocarbons)	+	NR	NR	NR	NR
Diesel, fuel marine	+	NR	NR	NR	NR
JP-10 synthetic jet fuel (exohexahydro-4,7-methanoindan)	+	NR	NR	NR	NR
RJ-5 synthetic jet fuel (hydrogenated dimers of norbornadiene)	+	NR	NR	NR	NR
JP-7 distillate jet fuel	+	NR	NR	NR	NR
JP-TS distillate jet fuel	+	NR	NR	NR	NR
Stoddard solvent	+	NR	NR	NR	NR
Tetralin	+	NR	NR	NR	NR
Hexachloroethane	+	NR	NR	NR	NR
Dimethyl methylphosphonate	+	NR	NR	NR	NR
Methyl isobutyl ketone	+	NR	NR	NR	NR
Methyl isoamyl ketone	+	NR	NR	NR	NR
Diisobutyl ketone	+	NR	NR	NR	NR
1,3,6-Tricyanohexane	+	NR	NR	NR	NR

NR, not reported.
[a]Based on cell counts from urine.

synthesize α_{2u} in the liver but do synthesize small amounts of the protein in the salivary gland.

Cellular and Molecular Mechanisms of α_{2u} Nephropathy

An early hypothesis for an association between α_{2u} and UG in the induction of the male-rat-specific nephropathy and associated carcinogenicity was that TMP and related hydrocarbons were being metabolized to their respective aldehydes, which formed a Schiff's base and covalently bound to α_{2u} (*16*). Whole-body autoradiographs after administration of [^{14}C] TMP demonstrated selective retention of radioactivity in the renal cortex of male but not female rats (*16*). Likewise, kidney cytosol contained prominent amounts of labeled TMP that exhibited a supralinear dose response, while no accumulation was evident in female kidney or in liver of either sex (*16*). Subsequent experiments demonstrated that the observed binding was not covalent, however (*17*). If kidney cytosol from male rats administered [^{14}C] TMP was separated by G75 Sephadex chromatography, the radioactivity coeluted with the low molecular weight protein fraction and free metabolites. If the cytosol was first dialyzed against phosphate buffer, radioactivity only coeluted with the low molecular weight proteins. When the protein denaturing detergent, sodium dodecyl sulfate, was added to the dialysis buffer, the radioactivity associated with the low molecular weight protein fraction was lost. These experiments demonstrated that the binding between the low molecular protein fractions and TMP was reversible, not covalent (*17*). Immunoassay of the same low molecular weight protein chromatography fractions demonstrated the presence of α_{2u}. These fractions were collected, extracted with acidified ethyl acetate, and analyzed by gas chromatography-mass spectrometry to identify the bound material. The reversibly bound metabolite of TMP was identified as 2,4,4-trimethyl-2-pentanol (TMPOH). Subsequent experiments exposed male F344 rats to 50 ppm TMP or 300 ppm UG and demonstrated that TMPOH was bound to α_{2u} (*18*).

Reversible binding to α_{2u} has been demonstrated for at least 13 chemicals in *in vitro* or *in vivo* studies (*17,19–21*). Even though the agents fall into rather diverse chemical classes, molecular modeling studies have demonstrated strong structure–activity relationships (*22*). Active chemicals fit deeply into a hydrophobic pocket of α_{2u}. When hydrogen bonding between the chemical and protein can

occur, the digestibility of α_{2u} by proteases is inhibited, leading to accumulation of the male-rat-specific protein in lysosomes of the P2 segment of the nephron (*23*).

The accumulation of α_{2u} is cytotoxic and results in single-cell necrosis (*24–26*). The exfoliated renal epithelium, which represents the nidus for granular cast formation, is restored by compensatory cell proliferation. This increase in cell proliferation is localized in the P2 segment of the nephron and to a much lesser extent in the P3 segment (*25,27,28*). Increased cell proliferation can be readily demonstrated using pulse (*27*) or continuous (*25–26,28*) administration of [^{3}H]-thymidine or bromodeoxyuridine, can be detected as early as 3 days after exposure to α_{2u}-inducing agents, and has been demonstrated to remain elevated through at least 50 weeks of exposure to UG (*25*). The increase in proliferation is dose related, with maximum tolerated doses (MTD) resulting in 5- to 12-fold greater numbers of labeled cells (*25,28*). Clear, no-effect doses have been demonstrated (*25,27,28*). Of great importance is the demonstration that kidneys of female rats that have been identically exposed to UG have no increase in cell proliferation (*25*). While this strongly suggests that the increase in cell proliferation requires the presence of large amounts of α_{2u}, a recent study comparing cell proliferation in d-limonene exposed F344 versus NBR male rats has shown that the protein is absolutely required (*26*). The NBR rat is the only identified strain of rat that does not synthesize the androgen-dependent form of α_{2u} (*29*). Whereas the F344 rats exposed to d-limonene exhibited a 5-fold increase in cell proliferation after 5 or 30 weeks of exposure to 150 mg/kg/day, NBR rats were unaffected (*26*). Both strains metabolized d-limonene to the 1,2-oxide, the nonmutagenic metabolite that reversibly binds to α_{2u} (*21,26,30*). The increase in cell proliferation associated with α_{2u} nephropathy is reversible. After exposures of up to 3 weeks to TMP or UG, proliferation returns to control rates within 1 week after cessation of exposure (*25*). Longer-term exposures result in a slower return to control rates. Morphologic evidence of regenerative tubules can still be identified 4 weeks after subchronic exposure ceases (*1,2*).

This sustained increase in cell proliferation is capable of promoting spontaneously initiated or chemically initiated cells of the proximal tubule to form preneoplastic and neoplastic lesions (*26,31*). The promoting activity is totally dependent on the presence of α_{2u}. When F344 rats were exposed to UG or TMP in an initiation–promotion study, concentration-related increases in preneoplastic and neoplastic renal lesions were evident in males initiated with ethylhydroxyethylnitrosamine (EHEN) and promoted with UG or TMP (*31*). No increases occurred in females. The promoting activity paralleled increases in cell proliferation (*25*). When NBR rats were initiated with EHEN and promoted with d-limonene, no increase in atypical tubules, atypical hyperplasia, or renal adenomas occurred (*26*). In contrast, F344 rats promoted with d-limonene developed increased numbers of atypical tubules and atypical hyperplasia, while F344 rats initiated with EHEN and promoted with d-limonene developed increased numbers of atypical tubules, atypical hyperplasias, and renal adenomas (*26*). Promotion of preneoplastic or neoplastic lesions only occurred in groups that also exhibited increased cell proliferation. An important observation from this study was the presence of occasional preneoplastic lesions in the kidneys of control rats of both strains, as these lesions are thought to represent precursors of spontaneous kidney tumors. The incidence of these lesions was increased by exposure to EHEN in both strains and by exposure to d-limonene alone in male F344 rats. These data strongly suggest that agents that cause α_{2u} nephropathy, such as UG, induce renal tumors in male rats through sustained increases in cell proliferation. The higher rate of cell proliferation decreases the amount of time available to repair DNA damage, increasing the probability of mutations leading to spontaneously initiated renal epithelial cells and promoting clonal expansion of such cells, thereby increasing the probability of neoplasia (*32,33*).

Additional evidence for the sex and species specificity of this syndrome comes from studies on levamisole. Levamisole, a drug used as an antihelminthic in cancer chemotherapy and in the treatment of rheumatoid arthritis in humans, causes α_{2u} nephropathy in male rats (*34*). No increase in urinary *N*-acetyl β-glucosaminidase, an indicator of nephrotoxicity, was present in patients receiving 150 mg levamisole per day for 26 weeks (*35*). Levamisole has not been studied yet for carcinogenicity in animals or humans.

Several of the chemicals that induce α_{2u}-related male rat kidney tumors also cause tumors in mouse liver. 1,4-Dichlorobenzene, isophorone, pentachloroethane, tetrachloroethylene, and UG represent such examples (*36–41*). The mechanism responsible for the induction of liver tumors is not definitively known at this time. Marked decreases in uterine cystic hyperplasia and uterine involution were apparent in the high-dose group of the UG mouse carcinogenicity bioassay but not in the middle- or low-dose groups (*42*). This suggests that the hormonal status of the high-dose female mice was affected by exposure to UG. It is well documented that either castration or administration of testosterone to female mice increases the incidence of hepatic neoplasia (*43*). Thus, the increased incidence of hepatic tumors in the UG mouse bioassay may have been due to a secondary mechanism that does not extrapolate to low exposures. Additional research will be necessary to further delineate the role of hormonal alteration for UG and the other chemicals known to cause α_{2u} nephropathy and female mouse liver tumors.

Not all agents that induce α_{2u} nephropathy have resulted in an increased incidence of kidney tumors in male rats. In some cases, this has been due to an inadequate length of exposure. For example, a series of hydrocarbons was evaluated in rats exposed for 90 days and held for an additional 19 months (*44,45*). None of the hydrocarbons caused increases in renal tumors. When rats were exposed to the same hydrocarbons for 1 or more years, tumors were induced in the kidneys of male rats. An antiepileptic agent has recently been shown to induce α_{2u} nephropathy in Wistar rats, but it did not induce renal neoplasia (*46*). Although α_{2u} was demonstrated immunohistochemically,

no information on binding, cell proliferation, or initiation/promotion is available. The incidence of renal tumors induced by the α_{2u} mechanism is much lower (0–26%) than can be achieved by genotoxic renal carcinogens administered at their maximum tolerated dose (*8*). Furthermore, the extent of mechanistic research available for different agents varies markedly (Table 1). Thus, although the weight of evidence available for this class of agents supporting this sex- and species-specific mechanism is very strong, determination of the relevance of this mechanism for a specific chemical's carcinogenic activity requires a case-by-case analysis.

Species Differences in Urinary Protein

Having established that the presence of α_{2u} is mandatory for the formation of male-rat kidney tumors after treatment with α_{2u} nephropathy-inducing agents, the question arises whether extrapolation of such carcinogenicity data to other species, including humans, is warranted. Most compounds that are carcinogenic in animals are generally assumed to pose some risk to humans. In the case of α_{2u} nephropathy-inducing agents, however, the carcinogenic mechanism is clearly associated with the presence of a specific urinary protein (α_{2u}) not found in humans or any other species. Several proteins sharing some amino acid sequence homology with α_{2u} have been identified in the serum and urine of various species, including humans (*47–50*). The presence of these partially homologous proteins in humans raised concern as to the possible interaction of these low molecular weight proteins with α_{2u} nephropathy-inducing agents, thus questioning the male-rat specificity of α_{2u} nephropathy. Assuming that a homologous protein reversibly binds the previously mentioned chemicals, the protein needs to be excreted into the plasma in large amounts, freely filtered by the glomerulus, readily reabsorbed in the proximal tubules, and catabolized in the lysosomes of the proximal tubule epithelial cells at a slower rate than normal upon binding one of the chemicals in order to induce similar lesions to α_{2u} nephropathy.

Based on estimated daily average urine production and body weights of rats and humans, Olson et al. (*51*) showed that rats excrete approximately 90 times more total protein than humans. Of the total protein excreted, the predominant fraction in rats consisted of low molecular weight proteins (18 kD), whereas a predominance of high molecular weight proteins (66 kD) was found in humans. The small amount of low molecular weight proteins excreted by male humans was identified as α_1-acid glycoprotein, α_1-microglobulin, myoglobin, and β_2-microglobulin. Of these four proteins, only α_1-acid glycoprotein and α_1-microglobulin share amino acid sequence homology with α_{2u} (*47,48,50*). α_1-Acid glycoprotein (AGP) and α_1-microglobulin (AMG) are synthesized in the liver of rats and humans (*50,52–54*), have been purified from the urine of rats and humans, as well as the urine of rabbits and guinea pigs in the case of AMG (*48,54*), and, thus, permit a direct comparison with α_{2u}.

If AGP and AMG reversibly bind α_{2u} nephropathy-inducing chemicals and/or their metabolites with the same affinity as α_{2u}, then one would expect that female rats, male NBR rats, rabbits, and guinea pigs would also develop renal disease after treatment with these chemicals. However, male NBR rats, female rats, and guinea pigs do not accumulate protein in the renal cortex after treatment with α_{2u} nephropathy-inducing agents and, thus, are refractory to this disease (*5,27,55,56–60*). Furthermore, several members of the superfamily, including AGP, have been shown not to bind α_{2u}-inducing metabolites (*61*). In addition, mice, which excrete comparable amounts of the low molecular weight mouse urinary protein (MUP) having the greatest amino acid sequence homology to α_{2u} (approximately 90%) (*6*), do not develop the protein-related nephropathy or renal tumors after chronic exposure to α_{2u} nephropathy inducing agents (*36,39–41,62–63*). Lehman-McKeeman et al. demonstrated that the lack of responsiveness of the mouse is due to a lack of α_{2u} nephropathy-inducing metabolites to bind to MUP and the lack of MUP resorption by mouse renal tubules (*64*).

Recently, a sex-linked human protein of similar size was identified in urine from patients with renal disease (*65,66*). This protein has been named urine protein 1 and has been called the human equivalent of α_{2u} (*65*). This reference has led to considerable confusion and miscitation. Jackson and Turner (*67*) purified and partially sequenced human urine protein 1 and determined that it is related to rabbit uteroglobin, not α_{2u}. Urine protein 1 does not bind d-limonene-1,2-oxide or 2,4,4-trimethyl-2-pentanol, two metabolites of chemicals that bind to α_{2u} (*61*). Urine protein 1 also is present in human urine at concentrations four to five orders of magnitude less that that of α_{2u} in male rat urine (*7*).

Conclusions

A detailed understanding of the mechanisms involved in α_{2u} nephropathy and renal carcinogenesis has been elucidated by investigating several chemicals in various animal, biochemical, and molecular modeling systems. All of the experimental data are consistent with the hypothesis that reversible binding of chemicals or their metabolites to this abundant male-rat-specific protein is causally related to the induction of disease. Our present understanding of this disease process strongly suggests that it is unlikely that nongenotoxic chemicals that have been shown to only induce renal tumors in male rats via this mechanism pose a carcinogenic risk to humans.

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